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<b>(21) International Application Number:</b> PCT/US97/12980 <b>(22) International Filing Date:</b> 24 July 1997 (24.07.97) <b>(30) Priority Data:</b> 08/686,796                      26 July 1996 (26.07.96)                      US <b>(71) Applicant:</b> GALLO CLINIC AND RESEARCH CENTER [US/US]; Building 1, Room 101, 1001 Potrero, San Francisco, CA 94110 (US). <b>(72) Inventors:</b> GORDON, Adrienne, S.; 23 Norwood Avenue, Kensington, CA 94707 (US). DIAMOND, Ivan; 761 San Diego Road, Berkeley, CA 94707 (US). DOHRMAN, Doug, P.; 1412 29th Avenue, San Francisco, CA 94122 (US). <b>(74) Agents:</b> STARK, Jon, R. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).		<b>(81) Designated States:</b> AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GH, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DETECTION OF CELLULAR EXPOSURE TO ETHANOL  <b>(57) Abstract</b>  <p>The present invention relates to determinable effects of ethanol exposure on the cellular localization and abundance of specific proteins, referred to herein as ethanol indicative proteins. More specifically, the present invention is based, in part, on the discovery that the catalytic C<math>\alpha</math> subunit of cAMP dependent protein kinase (PKA), which is normally localized in the Golgi apparatus area, appears to translocate to the nucleus upon exposure of a cell to ethanol. The present invention is further based on the observation that the <math>\delta</math>-subunit of PKC translocates from the Golgi area to the perinucleus and the nucleus in response to ethanol exposure, while the <math>\epsilon</math>-subunit of PKC migrates from the perinucleus into the cytoplasm. The present invention further relates to the discovery that the detectable amount of the regulatory subunit RI of PKA decreases, while the detectable amount of <math>\alpha</math>PKC, <math>\delta</math>PKC and <math>\epsilon</math>PKC increases upon exposure of a cell to ethanol. These discoveries provide the basis for assays that may be used to detect the exposure of cells to ethanol and assays that may be used for the screening of drugs or the development of treatments to modulate the effects of ethanol consumption. The invention further relates to kits for detecting the exposure of cells to ethanol. Kits of the invention may include antibodies, which preferably are labeled, capable of specifically binding to C<math>\alpha</math> or RI of the cAMP-dependent protein kinase, or, capable of specifically binding to <math>\alpha</math>PKC, <math>\delta</math>PKC or <math>\epsilon</math>PKC.</p>		

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DETECTION OF CELLULAR EXPOSURE TO ETHANOL

## I. FIELD OF THE INVENTION

The present invention is in the field of biological  
5 assays for the detection of ethanol exposure in mammals and  
for the evaluation of drugs that modify the cellular effects  
of ethanol consumption.

## II. BACKGROUND OF THE INVENTION

10 The abuse of ethanol remains a major public health  
problem in the U.S. and throughout the world. It is of  
interest to provide methods for the detection of ethanol  
exposure in individuals so as to monitor compliance with  
abuse treatment regimens. It is also of interest to provide  
15 assays useful for the evaluation of drugs that may be used to  
treat one or more of the adverse effects of chronic ethanol  
over-consumption. In order to provide such methods and  
assays, it is necessary to gain detailed understanding of the  
biochemical effects of ethanol exposure at a cellular level.  
20 Recent evidence suggests that ethanol modifies the function  
of certain proteins and signal transduction pathways, thereby  
producing changes in second messenger concentrations, protein  
kinases, and gene expression. This observation does not  
provide a specific test which enables the effects of ethanol  
25 to be readily determined. Recently, it has been shown that  
the specificity of protein kinases appears to correlate with  
their localization within the cell. Mochly-Rosen, 1995,  
*Science* 268:247.

Chronic alcoholism causes functional and pathologic  
30 changes in many organs, particularly the brain, but the  
molecular mechanisms which account for these effects are not  
well understood. It would be desirable to provide methods  
for monitoring the effect of chronic ethanol exposure on  
mammalian cells, and further desirable to provide methods for  
35 determining if an individual has been actively consuming  
ethanol for extended periods of time.

The present invention relates to an assay for detecting  
the effects of ethanol, particularly the chronic exposure of

ethanol, on animal cells, particularly human or other mammalian cells. This assay can be used both in a diagnostic test to determine ethanol consumption in an individual, or in screening for drugs or treatments which moderate, inhibit, 5 reverse or enhance the effects of ethanol consumption.

### III. SUMMARY OF THE INVENTION

The present invention relates, in part, to the discovery that exposure to ethanol alters dramatically the subcellular 10 localization of the catalytic  $\alpha$  subunit of the cAMP dependent protein kinase (PKA) and the  $\delta$ - and  $\epsilon$ -subunits of protein kinase C (PKC). For example, the catalytic  $\alpha$  subunit of PKA, which is normally localized to the Golgi apparatus area, appears to translocate to the nucleus upon 15 exposure of a cell to ethanol. Ethanol also has been shown to cause translocation of PKC activity from cytosolic to membrane fraction in astroglial cells and human lymphocytes and epidermal keratinocytes. The present invention further relates to the discovery that the detectable amount of the 20 regulatory subunit RI of PKA decreases, and the amounts of the  $\alpha$ -,  $\delta$ - and  $\epsilon$ -subunits of PKC increase in certain cell types, including but not limited to, NG108-15 cells ( $\alpha$ -,  $\delta$ -, and  $\epsilon$ -subunit) or PC12 cells ( $\delta$ - and  $\epsilon$ -subunit), upon the exposure to ethanol. These discoveries provide the basis for 25 assays that may be used to detect the exposure of cells to ethanol and further for assays that may be used for the screening of drugs or treatment to modulate the effects of ethanol consumption.

One aspect of the invention is to provide assays that 30 provide an indication of the exposure of a cell or an individual to ethanol by identifying at least one cell component, e.g., a protein, that has a cellular localization (distribution) that varies in correlation with the exposure of the cell to ethanol, and determining the distribution of 35 that cell component within a cell of a sample to be tested. In one preferred embodiment, the cell component comprises a subunit of the cAMP dependent protein kinase, PKA, the  $\alpha$

subunit being particularly preferred. In another preferred embodiment, the cellular component comprises an isozyme protein kinase C, PKC, wherein the  $\delta$  or the  $\epsilon$  isozyme of protein kinase C is particularly preferred.

- 5 Another aspect of the invention is to provide assays that provide an indication of exposure of a cell or an individual to ethanol by measuring the amount of protein that varies in amount in a relationship with the exposure of the cell to ethanol. In one preferred embodiment, the decrease  
10 of the detectable amount of the regulatory subunit RI of PKA in response to ethanol exposure is determined. In another preferred embodiment, the increase of the detectable amounts of  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC in response to ethanol exposure is measured.
- 15 Another aspect of the invention is to provide assays for screening therapeutic compounds that modulate the effects of ethanol on a cell. These screening assays measure the effects of a compound of interest to interfere with one or more of the cellular effects of ethanol described herein,  
20 i.e., changes in the localization of  $C\alpha$ ,  $\delta$ PKC, or  $\epsilon$ PKC, decreases in the amount of RI, increases in the amount of  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC, changes in the set of proteins phosphorylated by or expressed in response to  $C\alpha$ ,  $\delta$ PKC and  $\epsilon$ PKC. For example,  $C\alpha$  may induce phosphorylation of CREB and  
25 thereby its activation, resulting in the induction of CRE-regulated gene expression. Another aspect of the invention is to provide kits for detecting the exposure of cells to ethanol. Kits of the invention may include labeled antibodies capable of specifically binding to  $C\alpha$  or RI of PKA  
30 or to the  $\alpha$ -,  $\delta$ - and  $\epsilon$ -subunits of PKC, respectively.

#### IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A-1D shows micrographs indicating the location of the PKA catalytic subunit staining in NG108-15 cells after a  
35 forty eight (48) hour exposure to 200 mM ethanol.

FIGURE 2 is a graph showing the dependence of the percentage of cells exhibiting nuclear staining as compared

to Golgi staining on the concentration of ethanol to which the cells have been exposed.

FIGURE 3 is a series of micrographs comparing the movement of the C $\alpha$  subunit of PKA in cells exposed to ethanol against effects of treatment with various other agents as indicated.

FIGURE 4 shows the variation of the percentage of cells with Golgi staining over time, for cells exposed to ethanol, and for cells treated with forskolin.

10 FIGURE 5 shows Western blot analysis of the C $\alpha$  and RI PKA subunits in ethanol-exposed NG108-15 cells.

FIGURE 6 depicts immunohistochemical staining of  $\delta$ PKC in NG108-15 cells grown in defined medium in the presence or absence of EtOH or of PKC activation by PMA.

15 FIGURE 7 depicts immunohistochemical staining of  $\delta$ PKC in NG108-15 cells after a four (4) day exposure to 25 mM ethanol.

FIGURE 8 depicts immunohistochemical staining of  $\epsilon$ PKC in NG108-15 cells grown in defined medium in the presence or  
20 absence of EtOH or of PKC activation by PMA.

FIGURE 9 depicts immunohistochemical staining of  $\epsilon$ PKC in NG108-15 cells after four (4) day exposure to 25 mM ethanol.

## V. DEFINITIONS

25 As used herein, the following term(s), whether used in the singular or plural, will have the meanings indicated:

**Ethanol Indicative Protein.** As used herein, the term ethanol indicative protein refers to a gene product whose cellular localization or detectable amount changes in  
30 response to exposure to ethanol.

## VI. DETAILED DESCRIPTION OF THE INVENTION

The subject invention relates to discoveries concerning the effects of ethanol on the cellular localization and  
35 abundance of specific proteins as a consequence of exposure of cells to ethanol. Specifically, the invention relates to the discovery that the exposure of cells to ethanol induces

translocation of the  $C\alpha$  catalytic subunit of cAMP-dependant protein kinase (PKA) from the Golgi region to the nucleus. The invention relates further to the discovery that exposure of cells to ethanol induces translocation of the  $\delta$ -subunit of PKC from the Golgi region to the perinucleus and the nucleus, while inducing translocation of the  $\epsilon$ -subunit of PKC from the perinucleus to the cytoplasm. Additionally, the invention relates to the discovery that the detectable amount of type I (RI) regulatory subunit of PKA found in cells decreases in response to ethanol exposure, while the detectable amounts of  $\alpha$ PKC,  $\delta$ PKC, and  $\epsilon$ PKC increase in response to short term as well as long term exposure to ethanol. The cellular changes in response to ethanol exposure have numerous consequences beyond the effects of ethanol on cellular localization of  $C\alpha$  and  $\delta$ PKC and  $\epsilon$ PKC. For example, the dissociation of the catalytic subunit  $C\alpha$  from the regulatory subunit frees the  $C\alpha$  subunit to phosphorylate proteins. Furthermore, by translocating to the nucleus, the  $C\alpha$  subunit of PKA may phosphorylate a different set of proteins than those available in the Golgi apparatus or elsewhere in the cytoplasm. Moreover, the translocation of the  $C\alpha$  subunit may also alter the extent of phosphorylation of different proteins phosphorylated by  $C\alpha$ , such as CREB, as well as it may alter the CRE-regulated gene expression. The  $C\alpha$ -mediated changes in protein phosphorylation may also have detectable effects on gene expression; such PKA effects may also be used to monitor ethanol exposure. Similar effects of  $\delta$ PKC and  $\epsilon$ PKC translocation may be determined. Generally, proteins whose cellular localization or detectable amount are altered by ethanol exposure are referred to herein as ethanol indicative proteins.

In one aspect, the present invention provides a method for determining ethanol exposure of a sample containing at least one cell, comprising identifying a protein having a location that is substantially affected by exposure to ethanol, and determining the distribution of the protein within the cell, thereby producing an indication of the

exposure of the cell to ethanol. In one specific embodiment, the protein is the  $\alpha$  catalytic subunit of cAMP-dependant protein kinase (PKA). In another specific embodiment, the protein is the  $\delta$ - or  $\epsilon$ -isozyme of the protein kinase C (PKC).

- 5 The step of identifying preferably comprises staining the cell with a staining complex having specific binding affinity for the protein. The step of determining the location of the protein preferably includes imaging or observing the cell using conventional imaging techniques, e.g., a microscope.
- 10 Preferably, the sample contains a plurality of cells, and the determination is carried out for several of the plurality of cells. Preferably, the cells analyzed are derived from blood samples, e.g., lymphocytes, granulocytes, etc. Nuclear accumulation of the ethanol indicative protein, e.g., the  $\alpha$
- 15 of PKA,  $\delta$ PKC or  $\epsilon$ PKC may also be assessed by observing  $\alpha$ -,  $\delta$ PKC-, or  $\epsilon$ PKC-induced cellular events. For example, it may be possible to observe the chronic activation of CREB transcription factor and other nuclear substrates in response to  $\alpha$ -activation.
- 20 The methods of the invention may be employed to monitor withdrawal of ethanol from a subject. Specifically, after a chronic alcoholic withdraws from alcohol, it is expected that  $\alpha$  and  $\delta$ PKC leave the nucleus or nucleus and perinucleus, respectively, and return to the Golgi, while  $\epsilon$ PKC will return
- 25 from the cytoplasm to the perinuclear region. Thus, the techniques described may be used to monitor the withdrawal from ethanol over a relatively short period of time.

The invention provides methods for determining exposure of cells to ethanol comprising the steps of applying a stain

30 using specific affinity for the ethanol indicative protein, e.g., the  $\alpha$ -subunit of PKA, or the  $\delta$ - or  $\epsilon$ - PKC isozyme to the sample so as to identify the region or regions of the cell that contain  $\alpha$ ,  $\delta$ PKC, or  $\epsilon$ PKC, respectively. After the regions of the cell containing PKA or PKC have been

35 identified, the cell or cells are classified as to the distribution of the stain within the cells, wherein localization of  $\alpha$  stain in the cell nucleus,  $\delta$ PKC in the



perinucleus and the nucleus, and  $\epsilon$ PKC stain in the cytoplasm is indicative of prior exposure of the cell to ethanol. Cells that contain significant, i.e., greater than control cells, detectable amounts of stain for C $\alpha$  of PKA in the nucleus, the  $\delta$ -subunit of PKC in the perinucleus and nucleus, or  $\epsilon$ PKC in the cytoplasm, respectively, are indicative as being exposed to ethanol.

Advantageously, the determination of cellular localization of the ethanol indicative protein, e.g., C $\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC, comprises identifying first and second regions within each cell, and classifying cells as a first type if the protein is predominately present in a first region, and as a second type if the protein is predominately present in the second region. The number of cells of the first type may be compared to the number of cells of the second type for a determined number of cells within a sample. A number dependent on the proportion of cells of the first and second types, usually the ratio or percentage, may be correlated with a control derived from reference data to obtain a qualitative determination of whether exposure to ethanol of the sample has exceeded a certain threshold, or to obtain a semi-quantitative determination of the exposure to ethanol of the sample. In the case of C $\alpha$ , the first region will preferably be the nucleus of the cell, and the second region will preferably be the perinuclear Golgi apparatus. In the case of  $\delta$ PKC, the first region will preferably be the perinucleus and the nucleus of the cell, and the second region will preferably be the perinuclear Golgi apparatus. In the case of  $\epsilon$ PKC, the first region will preferably be the cytoplasm of the cell, and the second region will preferably be the perinucleus and the nucleus of the cell. The control will depend on the type of cells being examined and will usually be about 25-35%, i.e. if more than 25-35% of the cells display localization in the first region, the sample will be positive.

The classification step includes the step of identifying the cellular location of the stain and hence the location of

the ethanol indicative protein, e.g., the C $\alpha$ -subunit of PKA,  $\delta$ PKC or  $\epsilon$ PKC or any other proteins with comparable localization behavior. The precise means of identifying the cellular location of the ethanol-indicative protein, e.g.,

5 C $\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC will vary with label used in the stain. Generally, a variety of methods may be used for each type of label selected. For example, a radioisotope label may be detected through film (autoradiography), Charge Coupled Devices (CCDs) and the like.

10 When analyzing the results of the subject assays in which a sample containing a plurality of cells is stained with ethanol indicative protein, e.g., C $\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC-, specific stain, the percentage of cells that show altered location of the ethanol indicative protein must be  
15 considered. Not every ethanol exposed cell in a sample will show altered location of the ethanol indicative protein. However, significantly more cells with altered location of the ethanol indicative protein will be found in multiple cell containing samples that have been exposed to ethanol as  
20 opposed to control samples. Furthermore, the percentage of cells showing altered location of the ethanol indicative protein, i.e., translocation to the nucleus in the case of C $\alpha$ , translocation to the perinucleus and the nucleus in the case of  $\delta$ PKC, or translocation to the cytoplasm in the case  
25 of  $\epsilon$ PKC, is expected to increase with increasing duration of exposure to ethanol and with increasing amount of ethanol to which the cells are exposed. Statistical analysis may be used to develop quantitative correlations between the percentage of cells in a sample sharing altered location of  
30 the ethanol indicative protein and the amount of exposure. Other factors to consider when making such correlations include the age and condition of the source of the cell sample, the particular cell type being analyzed, and the like. The sample may be taken from a live subject, for  
35 example a human whose ethanol consumption is to be measured. Alternatively, cells cultured *in vitro* may be used in those embodiments of the invention that are directed to the

monitoring of ethanol in subjects, e.g., screening for therapeutic agents. Where the sample is taken from a live subject, the sample is preferably a blood-sample, containing nucleated cells, such as granulocytes and lymphocytes.

5

The ethanol consumption of a subject, particularly a human, could in principle be determined by assaying the cells obtained from the subject. Cells for analysis in the subject assays for ethanol exposure may come from a variety of  
10 locations within the body. Cell containing samples may be obtained from organs or non-organ tissue. Preferably, cell containing samples are obtained from easily removed tissues such as blood and skin. Because of the transient and reversible effects of ethanol on ethanol indicative proteins,  
15 e.g.,  $\alpha$ ,  $\delta$ PKC,  $\epsilon$ PKC, it is important that samples be analyzed with the assays of the invention as soon as possible after the sample is removed from a subject for analysis. Localization of ethanol indicative proteins such as the  $\alpha$  subunit of PKA,  $\delta$ PKC, or  $\epsilon$ PKC in granulocytes and/or  
20 lymphocytes can be investigated. Both of these cell types can be conveniently obtained from blood samples. To determine the effect of ethanol consumption in a particular individual, a comparison can be made between the proportion of cells having, e.g., predominately nuclear localization of  
25  $\alpha$ , perinuclear and nuclear localization of  $\delta$ PKC, or cytoplasmic localization of  $\epsilon$ PKC, to that obtained from a reference sample. To monitor progress of the individual over time, a number of samples can be taken, and variations in the localization of staining can be monitored. The technique can  
30 be used to determine the effects of a treatment on a live subject, by monitoring changes in the subject when provided with the treatment.

In another aspect, the present invention provides a method of measuring the exposure to ethanol of a sample  
35 containing at least one cell, the method comprising measuring, either quantitatively or qualitatively, the amount of a protein or polypeptide, which amount is dependent on

exposure of the cell to ethanol, and determining the amount of the protein in the cell. In one preferred embodiment, the polypeptide may be the type I regulatory subunit (RI) of PKA where a reduction, for example a 20% to 50% reduction  
5 compared to control cells not exposed to ethanol, is indicative of ethanol exposure. Alternatively, the detectable amount of the protein heat stable protein kinase inhibitor (PKI) may be measured and correlated with ethanol exposure. In another preferred embodiment, the increase of  
10 the detectable amount of the  $\alpha$ -,  $\delta$ -, or the  $\epsilon$ -subunit of PKC may be determined and correlated with ethanol exposure. The determination may be carried out by any of a variety of measurement methods well known to persons of ordinary skill in the art of molecular biology, such methods include ELISA,  
15 radioimmunoassay, western blot analysis, and the like.

The methods described herein may be used in screening drugs for their efficacy in moderating the effects of ethanol by measuring the effects of ethanol on a sample treated with the drug and exposed to ethanol, and comparing the results  
20 with results obtained from a control sample exposed to ethanol but not treated with the drug. The methods may also be used for providing an indication of the effects of ethanol consumption in a subject individual by comparing the results obtained with reference results, for example results obtained  
25 from a control subject, either *in vitro* or *in vivo*, or to other measurements taken from the same subject over a period of time to determine progress of alcoholism or of treatments. Numerous other applications will become apparent to those skilled in the art.

30 Using known immunocytochemistry techniques, a stain specific for the ethanol-indicative protein, e.g. the  $C\alpha$  catalytic subunit of PKA,  $\alpha$ PKC,  $\delta$ PKC or  $\epsilon$ PKC is prepared. Stains specific for RI and other proteins having cellular locations or quantities that may be correlated with ethanol  
35 exposure may be similarly prepared and used for the quantitative detection of, i.e., RI. The stain comprises a specific binding substance which binds specifically to the

targeted ethanol indicative protein, e.g., the C $\alpha$  subunit of PKA,  $\alpha$ PKC,  $\delta$ PKC,  $\epsilon$ PKC, RI, for example an antibody and a labelling moiety. Suitable antibodies may be prepared using conventional antibody production techniques. The antibodies  
5 may be monoclonal or polyclonal. The antibodies may also be obtained from genetically engineered hosts or from conventional sources. Antibodies may be prepared in response to the ethanol indicative protein, e.g., C $\alpha$ ,  $\alpha$ PKC,  $\delta$ PKC,  $\epsilon$ PKC, or RI, or immunologically cross-reactive fragments  
10 thereof. Techniques for antibody production are well known to the person of ordinary skill in the art, examples of such techniques can be found in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1988), Birch and Lennox, Monoclonal  
15 Antibodies: Principles and Applications, Wiley-Liss, New York (1995). The labelling moiety will be visibly observable in conventional immunohistochemical detection techniques being, for example, a fluorescent dye such as fluorescein, a radioisotope, a colloidal label, such as colloidal gold or  
20 colored latex beads, an enzyme label, or any other known labelling complex. Such stains may be prepared by conventional techniques, for example as described in Manson, Immunochemical Protocols: Methods in Molecular Biology Vol. 10, Humana Press, Totowa, NJ (1992), Beesley,  
25 Immunocytochemistry: A Practical Approach, IRL Press, Oxford, England (1993), the disclosure of which is herein incorporated by reference.

In the above described embodiments, methods have been explained for examining the effects of ethanol and  
30 therapeutic agents which affect these either *in vivo* or *in vitro*, and it will be readily apparent to one skilled in the art that these techniques may be applied to a number of problems. Determination of the localization of the C $\alpha$ -subunit of PKA,  $\delta$ PKC,  $\epsilon$ PKC or any other ethanol indicative  
35 protein under investigation described above has been carried out manually, by visual observation. This procedure may be automated, for example by computer-based image recognition,

or assays may be developed in which the localization of the protein is determined without visualization, for example by using reagents specific to the protein in combination with reagents having specific affinity for particular locations  
5 within the cell. Furthermore, the invention is not intended to be limited to the detection of the proteins mentioned above, but one skilled in the art may use other proteins whose behavior in the presence of ethanol is similar to those described above. Accordingly, the scope of the invention is  
10 not to be limited to the described embodiments, but is to be construed as incorporating all such variants which do not depart from the spirit thereof.

Another aspect of the invention is to provide methods for detecting the effects of ethanol on cells by measuring  
15 the phosphorylation of proteins that are differentially phosphorylated by, e.g.,  $C\alpha$  in the presence and absence of ethanol. As previously discussed, exposure of cells to ethanol results in the translocation of  $C\alpha$  to the nucleus where the  $C\alpha$  catalytic subunit may phosphorylate  
20 (serine/threonine targets) a set of proteins that differs from the set of proteins available for phosphorylation in the cytoplasm, plasma membrane or Golgi. The identity of such proteins that are differentially phosphorylated in response to ethanol exposure may readily be determined using  
25 conventional assay techniques known to the person of ordinary skill in the art of molecular biology. For example, radioactively labeled phosphate may be added to cultured cells grown in both the presence and absence of ethanol. Proteins from the labeled cells may then be extracted and  
30 separated on a one or two dimensional gel system. Isolated phosphorylated proteins may then be visualized by autoradiography and related techniques. After separation and visualization, changes in the level of phosphorylation of different proteins may be determined by comparing the results  
35 obtained from cells exposed to ethanol with the results obtained from cells not exposed to ethanol. For example, proteins that are differentially phosphorylated by  $C\alpha$  in

response to ethanol may be identified, e.g., by amino terminus amino acid residue sequencing. Proteins that are differentially phosphorylated by  $C\alpha$  in response to cellular ethanol exposure may be used in assays for the exposure of  
5 cells to ethanol. Furthermore, these differentially phosphorylated proteins may be used as the targets when screening for compounds that modulate the cellular effects of ethanol. Such assays include assays involving the steps of measuring the phosphorylation of differentially  
10 phosphorylated proteins. Compounds could be screened by measuring their effects on phosphorylation of these differentially phosphorylated proteins. Cells for use in the subject screening assays for compounds that modulate the effects of ethanol may be primary cells derived directly from  
15 a subject or may be cells from a cell line. Such assays include assays in which  $C\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC cellular localization is measured and assays in which the quantity of RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC is measured. Preferably, the cells used in the assay are from cell line cells, more preferably  
20 the cells are from a neuroblastoma cell line. Cell line cells are preferred for use in the subject screening assays because they provide consistency between assays. Cells for use in the assays of the invention may be obtained from cells cultured in vitro and include cells derived from brain or  
25 neural tissue, particularly NG108-15 neuroblastoma X glioma cells.

Another aspect of the invention is to provide kits for carrying out the subject methods. Kits generally contain one or more reagents necessary or useful for practicing the  
30 methods of the invention. Reagents may be supplied in pre-measured units so as to provide for uniformity and precision in test results. Kits for determining the exposure of cells to ethanol by means of determining the intracellular localization of  $C\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC comprise a stain specific  
35 for  $C\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC. Such kits may further comprise one or more of the following items: additional reagents required for the detection of  $C\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC that has complexed with the

stain, positive controls, negative controls, equipment for obtaining tissue samples, and the like. The invention also provides kits for determining the exposure of cells to ethanol by means of measuring the amount of RI,  $\alpha$ PKC,  $\delta$ PKC, 5 or  $\epsilon$ PKC. These RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC measurement kits comprise a stain specific for RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC. The RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC measurement kits may further comprise one or more of the following items: additional reagents for the detection of RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC complexed with the 10 stain; positive controls for RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC; negative controls for RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC; RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC solutions of known concentration; equipment for obtaining tissue samples, and the like. The invention also provides kits for testing compounds for their ability to 15 modulate cellular responses to ethanol using the assay methods of the invention. Such kits are essentially the same as the kits described above for the measurement of  $C\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC cellular localization and RI,  $\alpha$ PKC,  $\delta$ PKC, or  $\epsilon$ PKC levels; however, such kits may further comprise a cell line 20 useful for the detection of changes in  $C\alpha$ ,  $\delta$ PKC or  $\epsilon$ PKC localization.

Exemplary experimental procedures for detecting localization of the  $C\alpha$  catalytic subunit of PKA, and the  $\delta$ - and  $\epsilon$ -subunit of PKC are described below, but these 25 procedures are not critical, and other techniques will be apparent to those skilled in the art. These examples are offered by way of illustration and should not be construed as limitations of the invention.

## 30 VII. EXAMPLES

### A. Example 1: Ethanol Induced Translocation Of The $C\alpha$ Catalytic Subunit Of PKA

NG108-15 cells were plated onto single chamber slides in a defined medium at a density of approximately 35 40,000 cells/slide. The techniques and media used for growing the cells are not critical, and are known to those skilled in the art. Suitable techniques are described in



skilled in the art. Suitable techniques are described in Gordon et al., 1986, *Proc. Natl. Acad. Sci USA* 83:2105. The cells were maintained for an additional forty eight (48) hours in the defined media or the defined media containing various concentrations of ethanol (e.g. 25, 50, 100, 200 mM ethanol). The media were replaced by fresh media (with or without ethanol) daily and the slides were wrapped in parafilm to prevent ethanol evaporation. The cells were fixed with methanol on a cooled surface for two (2) to three (3) minutes, and the slides were then immersed twice for five (5) minutes each in phosphate buffered saline (PBS) on ice. After that, the cells were incubated with blocking buffer (1% normal goat serum in PBS containing 0.1% Triton-X-100) at 4°C for six (6) to twelve (12) hours and then incubated with primary antibody solution for forty eight (48) hours at 4°C in a humidified chamber. The primary antibody solution was prepared from primary antibodies raised in response to Cα (available from Transduction Laboratories and other companies and Susan Taylor at University of California at San Diego) diluted in PBS containing 0.1% Triton X-100 and 2 mg/ml fatty acid free bovine serum albumin. The slides were washed as before and incubated in the appropriate FITC (fluorescein-isothiocyanate)-conjugated secondary antibody diluted in the same solution at 1:1000.

After twenty four (24) hours, the slides were washed and coverslipped using Vectashield™ mounting medium (Vector Labs). The images in FIG. 1A and FIG. 1B were made using a BIORAD™ 1024 confocal microscope. The images in FIG. 1C and FIG. 1D were made using a Leica™ DMBR microscope equipped with a fluorescein filter.

To determine reversibility of exposure to ethanol, results shown in FIG. 1C, the cells were exposed to media containing 200 mM ethanol for forty eight (48) hours then washed three times with fresh media containing no ethanol and incubated without ethanol for an additional forty eight (48) hours. To determine specificity of binding of the stain, results shown in FIG. 1D, 0.1 mg/ml purified catalytic

subunit was added to the primary antibody solution two (2) hours prior to incubation with the fixed cells.

To obtain the numerical data shown in FIGURE 2, random fields were selected on the slide and the cells within the 5 field classified as either having primarily Golgi staining or primarily nuclear staining for C $\alpha$ . Although using only two types of classification simplifies scoring of cells, it may be desirable to classify cells into a plurality of classification groups dependent on the degree of staining in 10 each of several locations. In some instances, it may even be desirable to provide continuous variable classification of each cell, for example, if image intensity is measured at a given point in a cell. At least five (5) fields were classified for a total of at least one hundred (100) cells 15 per slide. The observer was blind to the experimental condition of the slides. Data points are the mean  $\pm$  SEM (standard error of the mean copy) of four (4) experiments, \*p<0.05.

The results obtained can be summarized as follows:  
20 NG108-15 cells, forming a control, which had not been exposed to ethanol were stained with the specific stain. C $\alpha$  was found in the perinuclear Golgi area in approximately 80% of the control cells, as depicted in FIG. 1A. In the remaining 20% of the control cells, C $\alpha$  was found predominately in the 25 nucleus and cytoplasm. This localization of the C $\alpha$  subunit to the Golgi has been previously observed by Nigg and co-workers. Nigg *et al.*, 1985, *EMBO J.* 4:2801-2806. Cellular localization of C $\alpha$  was further confirmed by co-localization with Golgi-specific markers, including mannosidase II, and 30 ceramide. Nigg *et al.*, *supra*.

Other cell samples were exposed to ethanol of varying concentrations (25, 50, 100, 200 mM), for a period of forty eight (48) hours, and the assay repeated. The results are shown in FIGURE 2. As can be seen from FIGURE 2, 75% of cells 35 treated with 200 mM ethanol showed predominant localization of C $\alpha$  in the nucleus. A micrograph of these cells is shown in FIG. 3B. The results of these investigations serve as a

reference to which a sample of cells suspected of being exposed to ethanol can be compared. To provide a screen for a drug or therapeutic agent to discover whether it has any effect on the cellular effects of ethanol, the above  
5 procedure can be repeated with the drug present in the growth medium in addition to alcohol. Since 100 mM ethanol is found to have a noticeable effect, and 200 mM ethanol is found to have a marked effect on the localization of C $\alpha$  PKA, a screen for a drug can be provided employing a single growth medium  
10 preferably having a ethanol concentration of the order of at least 100 mM, and more preferably of 200 mM, or more. Of course, several growth media containing differing amounts of ethanol may be used, as described above, to investigate efficacy of the drug at varying levels of ethanol  
15 concentrations. This may be particularly useful for investigating the effects of a drug on long-term low-level exposure to ethanol.

The results from a control sample can be stored so that when screening for a particular drug, it is not necessary to  
20 conduct a control experiment each time. However, it is often desirable to conduct a control experiment whenever a drug is screened, to compensate for variations in other factors which may affect the results.

The above procedure is sufficient to form the basis for  
25 a screen. Additional factors having an effect on the localization of PKA C $\alpha$  and activity of other PKA subunits have been identified. The following information may therefore be of assistance in assessing how the screening method may be affected by external factors.

30 Reversibility of the localization is demonstrated by FIG. 1C which is a micrograph of a similar sample forty eight (48) hours after withdrawal from the ethanol. As can be seen, the majority of the C $\alpha$  had returned to the Golgi apparatus. In a screen for a drug, therefore, it is  
35 important to classify the cells relatively soon after removal from the medium, usually within forty eight (48), preferably within twelve (12) hours. Similarly, samples taken from

patients should be classified soon after removal.

To test for specificity of the stain, the stain was preabsorbed by purified C $\alpha$  prior to staining, and as can be seen from FIG. 1D, virtually no staining resulted, indicating  
5 that the polyclonal antibody stain is specific to C $\alpha$ .

The time dependence of the localization of the C $\alpha$  and the effects of other substances were investigated (FIG 3A-3D) as follows: NG108-15 cells were cultured as described above. After two (2) days in defined media, cells received media  
10 containing either 200 mM ethanol (FIG. 3B), 1  $\mu$ M forskolin (panel d), or 10  $\mu$ M PGE<sub>1</sub> (Prostaglandin E<sub>1</sub>) (FIG. 3C) for various lengths of time. Control cells received fresh media alone at the same time points. All slides were fixed four (4) days after plating and stained for C $\alpha$  as described above.  
15 Similar experiments were performed using 25 mM and 50 mM ethanol over a period of four (4) to five (5) days; comparable results were obtained.

To obtain the numerical results, depicted in FIGURE 4, fields were selected as described above, and cells were  
20 scored as either having C $\alpha$  staining confined primarily to the Golgi or extensive staining outside of the Golgi. Data points are the mean  $\pm$  SEM of three (3) experiments.

The results will now be discussed with reference to FIGS. 3A-3D AND FIG. 4. FIG. 3A shows that the C $\alpha$  was  
25 localized at the Golgi in the control cells, as before, and FIG. 3B shows that after forty eight (48) hours exposure to ethanol, C $\alpha$  staining was found in the nucleus. This confirms the earlier described results.

Stimulation by 10  $\mu$ M PGE<sub>1</sub> resulted in diffuse staining  
30 throughout the cell, as shown in panel FIG. 3C. Similar results were achieved by treatment with 1  $\mu$ M forskolin, as shown in FIG. 3D. Maximal translocation of C $\alpha$  away from the Golgi occurred after about thirty (30) minutes, with forskolin or PGE<sub>1</sub>, which is when the micrographs shown

35

in FIG. 3C and 3D were taken. This was followed by desensitization and a return of staining to the Golgi.

This is clearly contrasted with the effects of exposure to ethanol, where (see FIGURE 4) after a relatively brief exposure to ethanol (from 30-60 minutes), little change in the localization of C $\alpha$  was detected. After six (6) hours exposure to ethanol, translocation from the Golgi to the nucleus of the C $\alpha$  was apparent, and after twelve (12) hours, most of the cells had developed prominent nuclear staining, with a corresponding decrease in Golgi staining. This staining remained through forty eight (48) hours of chronic exposure to ethanol.

Thus, in screening for the effect of drugs on ethanol uptake, best results will be obtained if the cells are left in the growth medium for at least about twelve (12) hours, and more preferably about forty eight (48) hours (two (2) days). The effects of ethanol exposure can be distinguished from the effects of other substances which produce a relatively temporary reversible change in C $\alpha$  localization.

The investigation of movement and activity of other PKA subunits particularly type I (RI) and type II (RII) regulatory subunits was investigated. Stains using monoclonal antibodies, prepared by conventional immunocytochemistry techniques, analogous to those described above for the C $\alpha$  subunit were used. No RII subunit was detected either by immunofluorescence or by Western blot analysis in NG108-15 cells, but the RI subunit was detected primarily on the Golgi apparatus. Ethanol was not found to have any significant effect on the localization of the RI subunit within the cell. It was, however, found that the amount of RI subunit was decreased by exposure to ethanol. Results of a Western blot analysis showing that exposure to 200 mM ethanol for forty eight (48) hours had no effect on the amount of C $\alpha$  subunit, but produced a decrease of about 40% (43 +/- 3%) in the RI subunit are shown in FIG. 5A-5B. Thus, an alternative assay procedure can be provided by measuring the amount of PKA RI.

With the above results, the effects of ethanol on NG108-15 cells can thus be clearly identified. To produce a screen for therapeutic agents or drugs that modulate the cellular effects of exposure to ethanol, these cells can be exposed to ethanol in the presence of a drug whose activity is to be investigated, and the results compared to those obtained from cells exposed to ethanol in the absence of the drug.

**B. Example 2: Ethanol Induced Translocation Of  $\delta$ PKC And  $\epsilon$ PKC**

Immunohistochemistry of  $\delta$ PKC in NG108-15 cells, grown in defined medium, shows predominant Golgi staining (FIG. 6A-6C); approximately 70% of these cells show Golgi staining (TABLE I). After chronic ethanol exposure (48 hrs., 200 mM EtOH),  $\delta$ PKC is localized to the perinucleus and nucleus and absent from the Golgi (FIG. 6A-6C and FIG. 7A-7C). More than 90% of the cells show perinuclear and nuclear staining (TABLE I). The specificity of the fluorescence staining for  $\delta$ PKC is indicated by the absence of staining when the anti- $\delta$  antibody is preabsorbed with the immunizing peptide before labelling of the cells (FIG. 7). These results suggest that chronic ethanol exposure causes translocation of  $\delta$ PKC from the Golgi to the perinucleus and nucleus since there is little  $\delta$ PKC remaining in the Golgi area (TABLE I) after chronic ethanol exposure.

Ethanol also alters localization of  $\epsilon$ PKC. In naive cells,  $\epsilon$ PKC is localized to the perinucleus in more than 90% of the cells (FIG. 8A-8C, FIG. 9A-9C and TABLE I), with no measurable cytoplasmic staining. After chronic ethanol exposure,  $\epsilon$ PKC staining is observed throughout the cytoplasm in greater than 90% of the cells (FIG. 8A-8C, FIG. 9A-9C and TABLE I); perinuclear staining is still present in more than 90% of the cells (FIG. 8A-8C, FIG. 9A-9C and TABLE I). The staining for  $\epsilon$ PKC appears to be specific since no staining is observed when the anti- $\epsilon$  antibody is preabsorbed with immunizing peptide (FIG. 9A-9C). Ethanol-induced altered localization of  $\delta$ PKC and  $\epsilon$ PKC is also observed after exposure

to 25 mM ethanol for four (4) days (FIG. 7A-7C and 9A-9C).

Ethanol-induced altered localization of PKC isozymes could be similar to that induced by phorbol esters or hormones or to sites different from these latter activators. Naive NG108-15 cells were, therefore, incubated in for ten (10) min. in 100 nM PMA, to then determine localization of  $\delta$ PKC and  $\epsilon$ PKC. On activation by PMA, the  $\delta$  isozyme is mainly translocated to the perinucleus (FIG. 6A-6C), suggesting that ethanol-induced translocation of  $\delta$ PKC is to sites similar to those occupied after activation by PMA. In contrast, translocation of  $\epsilon$ PKC due to PMA activation results in nuclear and perinuclear cytoplasm localization of this isozyme, different from ethanol-induced translocation to the cytoplasm (FIG. 8A-8C).

The effects of ethanol on NG108-15 cells can thus be clearly identified by determination of the localization of  $\delta$ PKC and  $\epsilon$ PKC. To produce a screen for therapeutic agents or drugs that modulate the cellular effects of exposure to ethanol, these cells can be exposed to ethanol in the presence of a drug whose activity is to be investigated, and the results compared to those obtained from cells exposed to ethanol in the absence of the drug.

25

TABLE I

30

$\delta$ PKC						$\epsilon$ PKC				
Golgi Staining staining (%) cell			Perinuclear staining (%) cell			Perinuclear staining (%) cell			Cytoplasm staining (%) cell	
Control	EtOH		Control	EtOH		Control	EtOH		Control	EtOH
74 $\pm$ 7	2 $\pm$ 2		2 $\pm$ 2	93 $\pm$ 5		95 $\pm$ 2	94 $\pm$ 3		5 $\pm$ 2	94 $\pm$ 2
(n=5)	(n=5)		(n=5)	(n=5)		(n=5)	(n=5)		(n=5)	(n=5)

35

\* The percentage of particular immunostaining was obtained from five (5) experiments, each experiment was counted in four (4) fields per slide, three (3) or four (4) different slides in the same experiment.

35

All patents, patents applications, and publications cited are incorporated herein by reference in their entirety.

The foregoing written specification is considered to be  
5 sufficient to enable one skilled in the art to practice the  
invention. Indeed, various modifications of the above-  
described methods for carrying out the invention which are  
obvious to those skilled in the field of cellular biology or  
related fields are intended to be within the scope of the  
10 following claims.

15

20

25

30

35



## CLAIMS

WHAT IS CLAIMED IS:

1. A method for determining the exposure to  
5 ethanol of a sample containing at least one cell having a plurality of cellular subregions, comprising:
  - (a) applying to said sample a stain having specific binding affinity for an ethanol indicative protein;
  - (b) identifying cellular subregions of said cell  
10 containing said stain; and
  - (c) classifying said sample according to the distribution of stain with respect to said cellular subregions of said cell compared to a control cell which has not been exposed to ethanol, wherein alteration of said  
15 distribution of stain compared to said control cell is indicative of prior exposure of the sample to ethanol.
2. The method according to Claim 1, wherein said plurality of cellular subregions includes first and second  
20 predetermined cellular subregions, and wherein said cell is classified as a first type if the stain is predominately present in said first predetermined cellular subregion, and as a second type if the stain is predominately present in said second cellular subregion.
- 25 3. The method according to Claim 2, wherein said cell has a nucleus and a Golgi apparatus, said first cellular subregion comprising the nucleus and said second cellular subregion comprising the Golgi apparatus of the cell.
- 30 4. The method according to Claim 3, wherein said first type corresponds to cells substantially affected by ethanol and said second type corresponds to control cells.
- 35 5. The method according to Claim 4, wherein said ethanol indicative protein is C $\alpha$  of PKA.

6. The method according to Claim 2, wherein said cell has a nucleus, a perinucleus, and a Golgi apparatus, said first cellular subregion comprising the nucleus and perinucleus, and said second cellular subregion comprising  
5 the Golgi apparatus of the cell.

7. The method according to Claim 6, wherein said first type corresponds to cells substantially affected by ethanol and said second type corresponds to control cells.  
10

8. The method according to Claim 7, wherein said ethanol indicative protein is  $\delta$ PKC.

9. The method according to Claim 2, wherein said cell has a perinucleus and a cytoplasm, said first cellular subregion comprising the cytoplasm and said second cellular subregion comprising the perinucleus of the cell.  
15

10. The method according to Claim 9, wherein said first type corresponds to cells substantially affected by ethanol and said second type corresponds to control cells.  
20

11. The method according to Claim 10, wherein said  
25 ethanol indicative protein is  $\epsilon$ PKC.

12. The method according to Claim 2, wherein said classifying comprises imaging said cell using a microscope.

13. The method according to Claim 2, wherein said sample contains a plurality of cells, the method further comprising repeating said classifying for a number of said plurality of cells, and obtaining a numerical measure of the exposure of said sample to ethanol dependent on the number of  
35 cells of each type and the total number of cells classified.

14. The method according to Claim 2, wherein said stain comprises an antibody having a detectable moiety and a specific binding affinity selected from the group consisting of PKA C $\alpha$ ,  $\delta$ PKC, and  $\epsilon$ PKC.

5

15. The method according to Claim 14, wherein said detectable moiety comprises fluorescein and a second antibody.

10 16. The method according to Claim 2, wherein said cell is derived from neural tissue.

17. The method according to Claim 2, wherein said cell is selected from the group consisting of granulocytes  
15 and lymphocytes.

18. The method according to Claim 2, wherein prior to said applying of step (a), said sample is exposed to ethanol in the presence of a therapeutic agent, and wherein  
20 the results of said classifying of step (c) are compared to results of classifying a sample control exposed to ethanol but not exposed to the said therapeutic agent, thereby providing an indication of said effect of the therapeutic agent on ethanol-mediated cellular effects.

25

19. A screening method for determining whether a test substance alters the effects of ethanol in mammalian cells, comprising:

(a) providing a sample containing a plurality of  
30 mammalian cells;

(b) providing a culture medium including ethanol;

(c) including said test substance in said culture medium;

(d) exposing said cells to said culture medium for  
35 a predetermined period of time;

(e) staining said cells with a stain having specific binding affinity for an ethanol indicative protein;

(f) classifying each cell according to the distribution of stain within the cell; and

(g) comparing the results of said classifying to  
5 distribution of stain in a control which has not been exposed to said test substance, wherein alteration of said distribution of stain compared to said control is indicative for effects of a test substance on the effects of ethanol in mammalian cells.

10

20. The screening method according to Claim 19, wherein said cells are classified into one of at least two categories, and wherein a numerical result dependent on the proportion of cells classified in a predetermined category is  
15 obtained, and the numerical result is compared to the numerical result obtained from the classification of the control cells.

21. The screening method according to Claim 19,  
20 wherein the cells are exposed to ethanol for at least twelve (12) hours.

22. The screening method according to Claim 19, wherein cells are exposed to ethanol for about two (2) days.  
25

23. The screening method according to Claim 21, wherein said cells are derived from neural tissue, each having a nucleus, a perinucleus, and a Golgi apparatus, and wherein cells are classified as substantially affected by  
30 ethanol if said stain is predominantly present in the nucleus or perinucleus.

24. The screening method according to Claim 23 wherein said ethanol indicative protein is C $\alpha$  of PKA and  
35  $\delta$ PKC.

25. A screening method according to Claim 24,  
wherein the cells are NG108-15 neuroblastoma X glioma cells.

26. The screening method according to Claim 25,  
5 wherein the ethanol is provided at a concentration of at  
least about 100 mM.

27. The screening method according to Claim 19,  
wherein said cells have a nucleus, a perinucleus, and a  
10 cytoplasm, and wherein said cells are classified as  
substantially affected by ethanol if said stain is  
predominantly present in the cytoplasm.

28. The screening method according to Claim 27  
15 wherein said ethanol indicative protein is  $\epsilon$ PKC.

29. The screening method according to Claim 28,  
wherein the ethanol is provided at a concentration of at  
least about 200 mM.

20

30. A method for determining the exposure to  
ethanol of a sample containing at least one cell, comprising:

(a) determining the amount of an ethanol  
indicative protein in said cell; and

25 (b) classifying the sample on the basis of the  
amount of said ethanol indicative protein in said cell  
compared to control cells which have not been exposed to  
ethanol.

30 31. The method of Claim 30, wherein said ethanol  
indicative protein is selected from the group consisting of  
PKA RI,  $\alpha$ PKC,  $\delta$ PKC, and  $\epsilon$ PKC.

32. The method according to Claim 31, wherein the  
35 amount of said ethanol indicative protein is determined by  
Western Blot analysis.

33. A method for determining the exposure of a subject to ethanol, comprising:

(a) providing a sample containing at least one cell from the subject;

5 (b) staining said sample with a stain having specific binding affinity for an ethanol indicative protein;

(c) classifying said sample according to the distribution of stain within said cell compared to a control which has not been exposed to ethanol, wherein alteration of  
10 said distribution of stain compared to said control is indicative of prior exposure of said sample to ethanol.

34. The method according to Claim 33, wherein said sample comprises a blood sample containing granulocytes and  
15 lymphocytes.

35. The method according to Claim 33, wherein said control is selected from the group consisting of (1) an earlier sample taken from the same subject, (2) a sample  
20 taken from a further subject having a determined exposure to ethanol, and (3) a sample comprising cells cultured in vitro and having a known exposure to ethanol.

36. A method for determining exposure to ethanol  
25 of a sample containing at least one cell having cell components, comprising:

(a) determining a property of a cell component said property being property dependent on exposure of the cell to ethanol comprising at least one of (1) localization,  
30 (2) amount, and (3) activity; and

(b) classifying the sample on the basis of said determining.

37. The method according to Claim 36, wherein said  
35 cell component is a protein.

FIG.1A

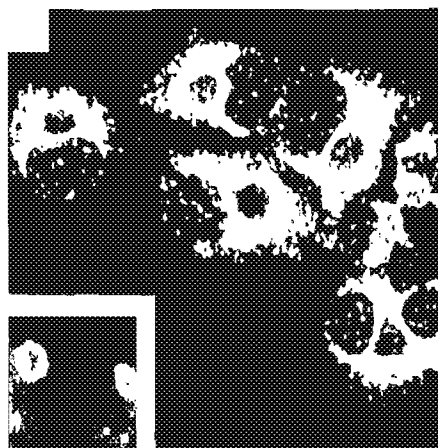


FIG.1B

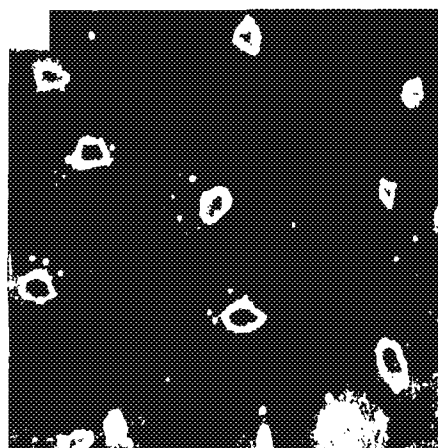
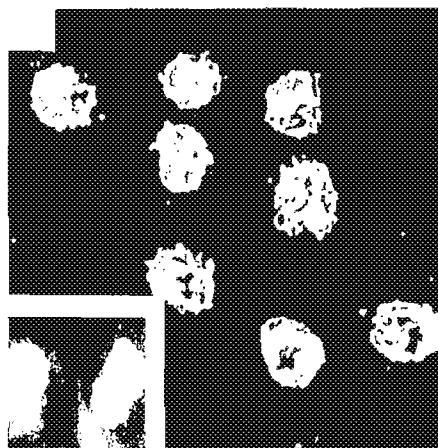


FIG.1C

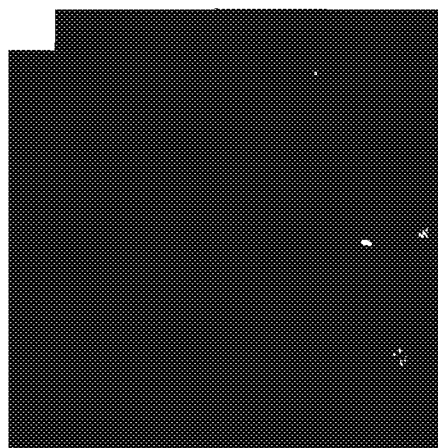


FIG.1D

2/9

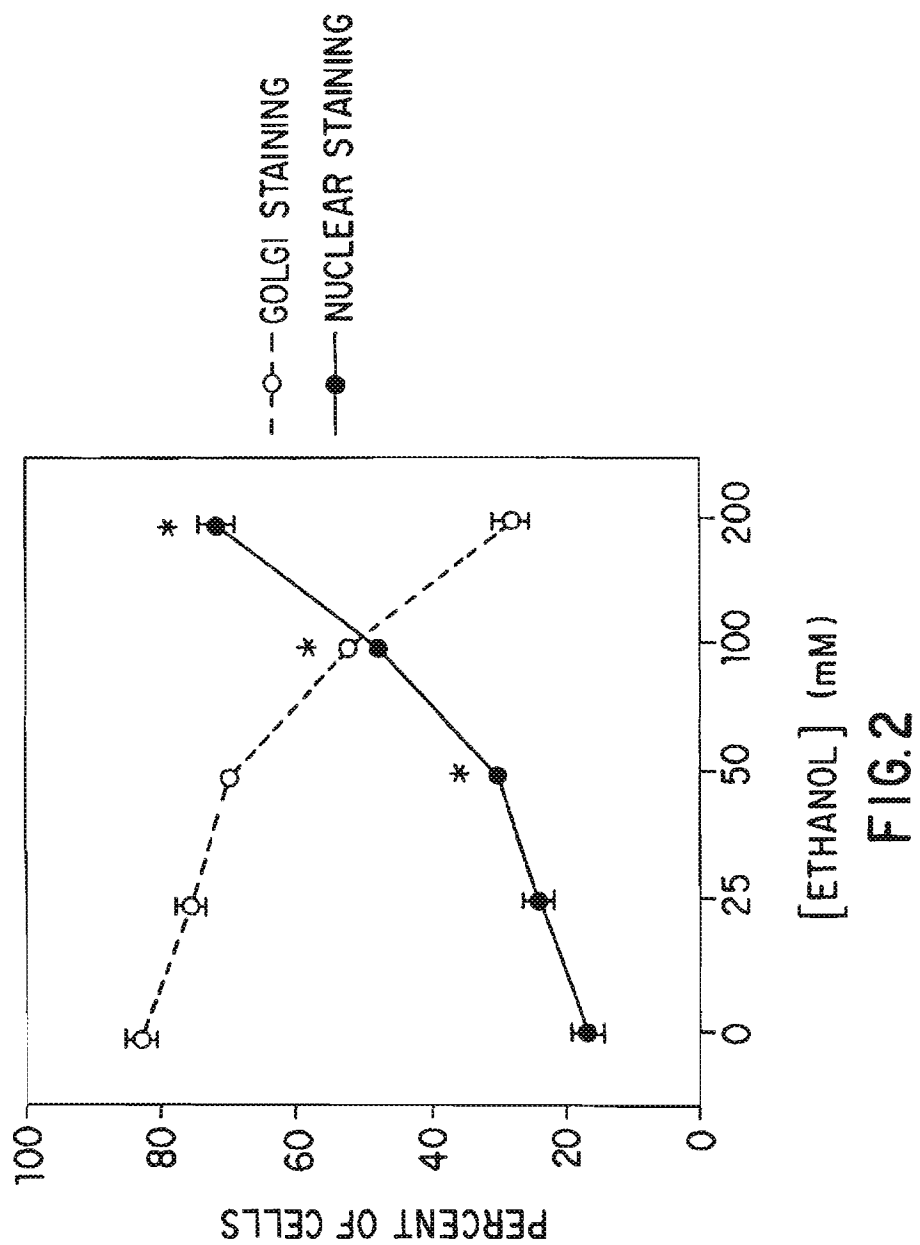


FIG.2



3/9

CONTROL

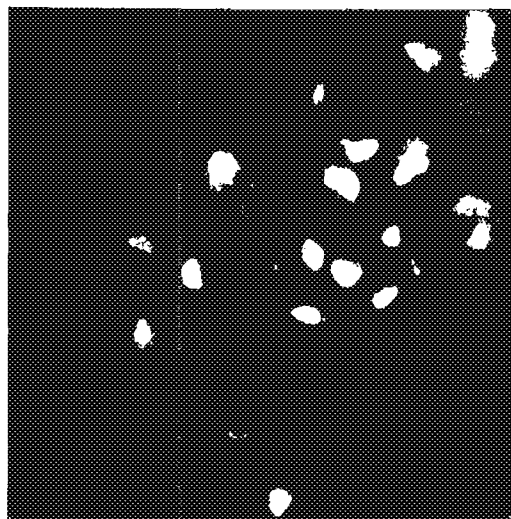


FIG.3A

ETHANOL

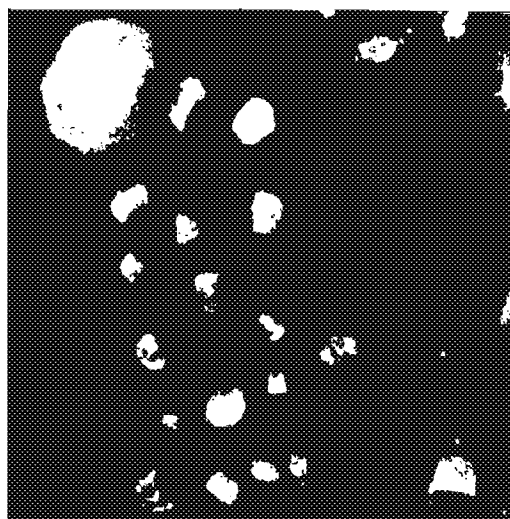


FIG.3B

PGE<sub>1</sub>

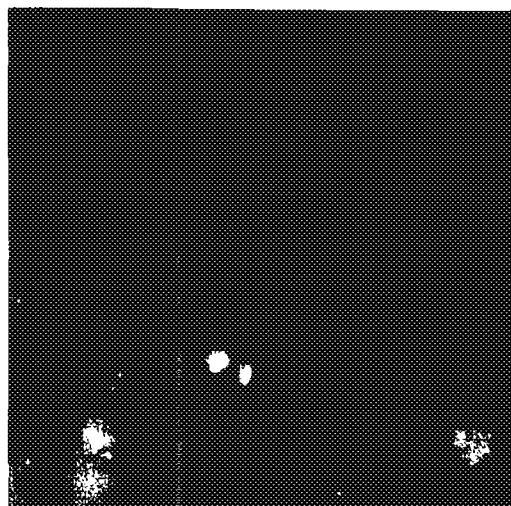


FIG.3C

FORSKOLIN

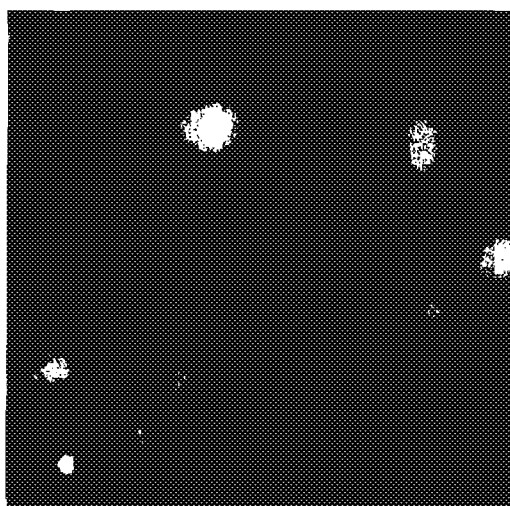


FIG.3D

4/9

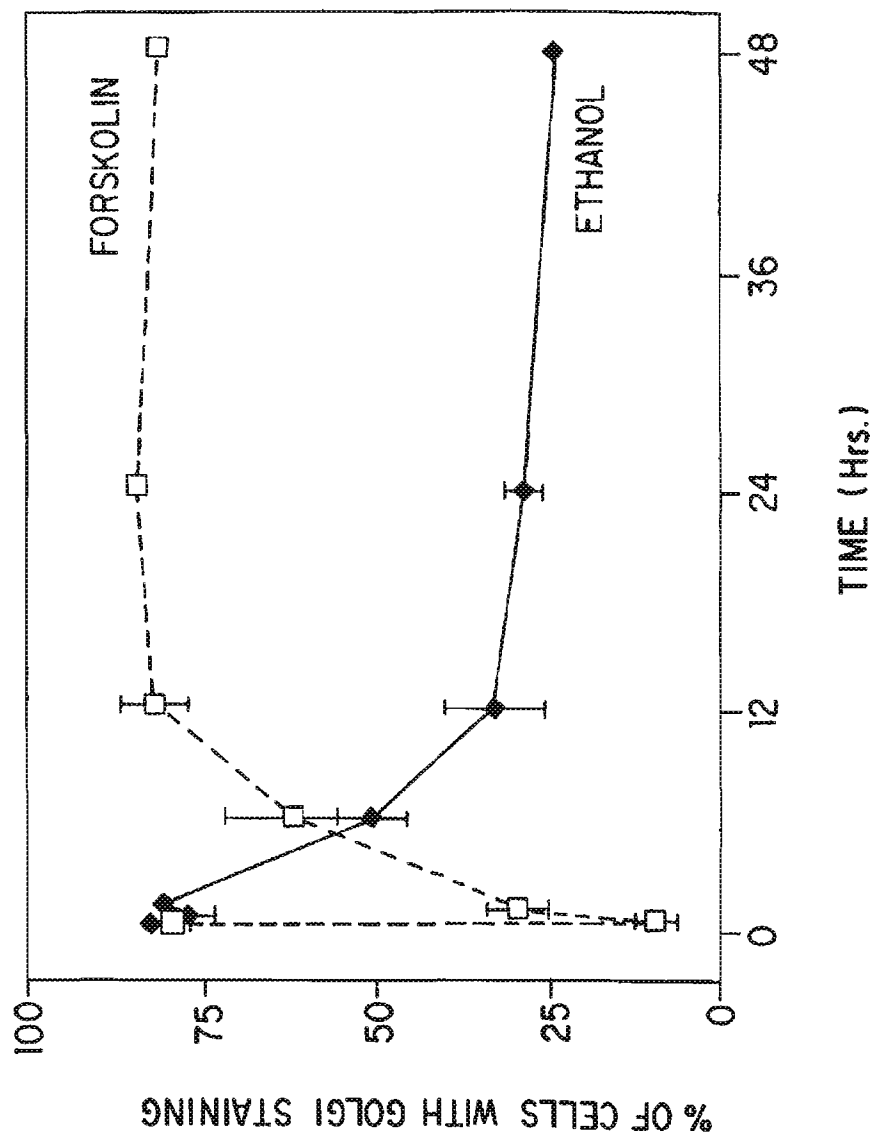


FIG. 4

5/9

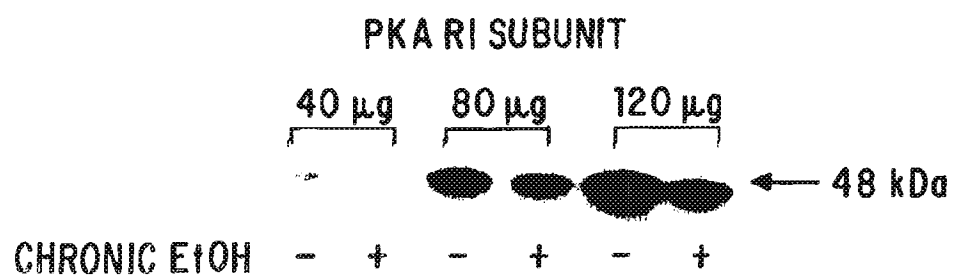


FIG. 5A

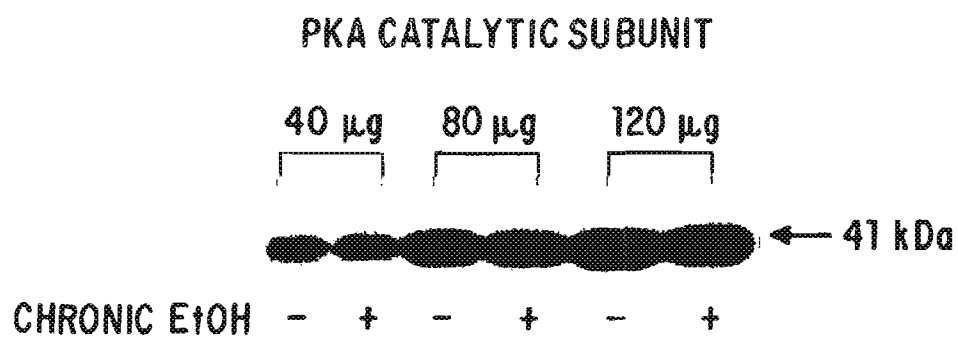


FIG. 5B

6/9  
PKC  $\delta$

CONTROL

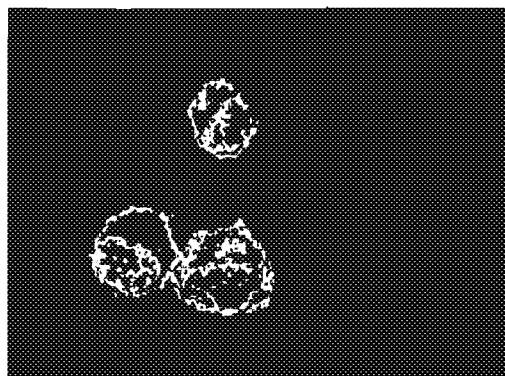


FIG.6A

EtOH  
200 mM

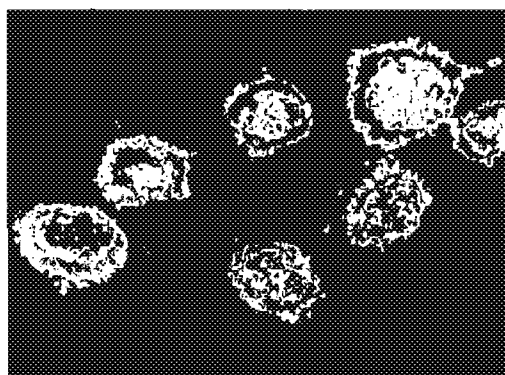


FIG.6B

PMA

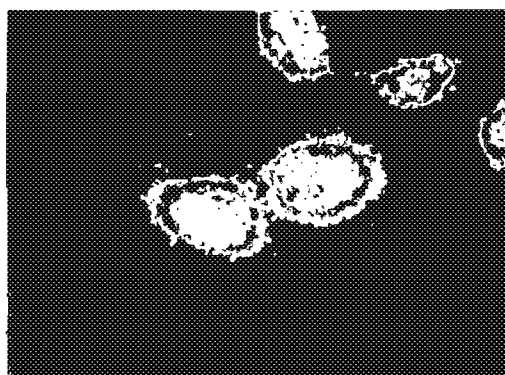


FIG.6C  
SUBSTITUTE SHEET (RULE 26)

7/9  
PKC  $\delta$

CONTROL

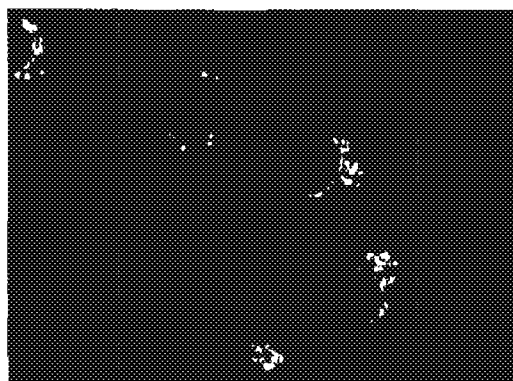


FIG.7A

EtOH  
25 mM



FIG.7B

Pre.

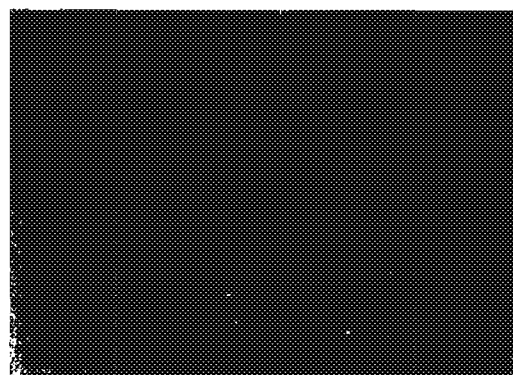
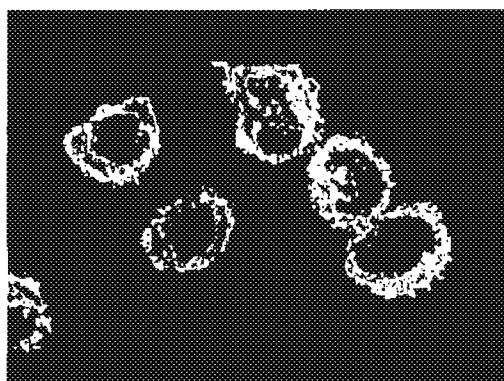


FIG.7C

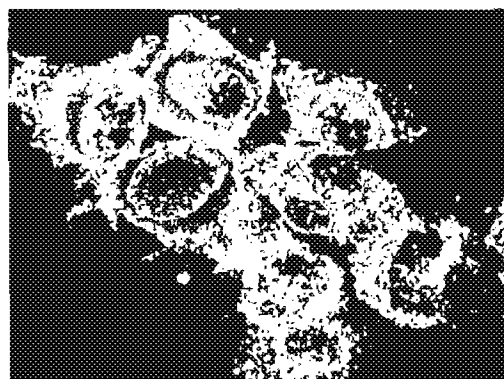
8/9  
**PKC  $\epsilon$**

**CONTROL**



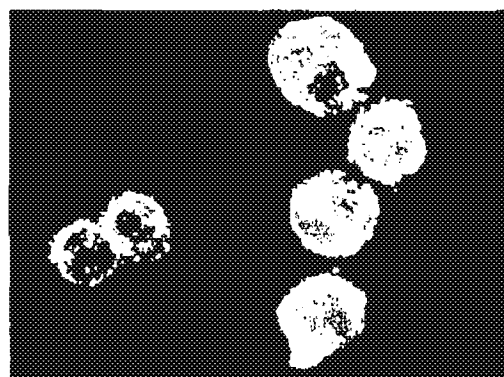
**FIG.8A**

**EtOH  
200 mM**



**FIG.8B**

**PMA**



**FIG.8C**

9/9  
PKC  $\epsilon$

CONTROL



FIG.9A

EtOH  
25 mM

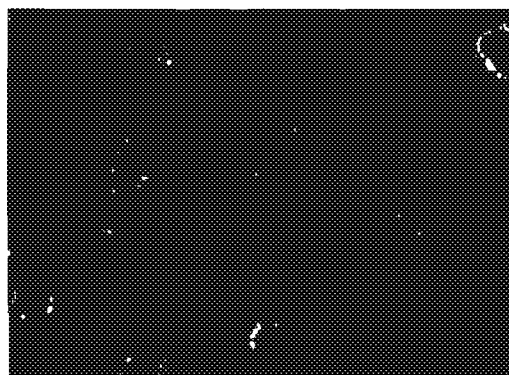


FIG.9B

Pre.

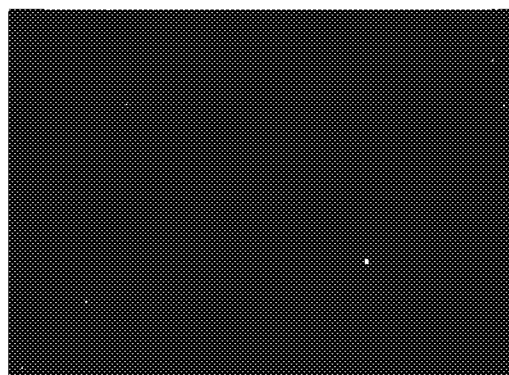


FIG.9C

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/12980

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : G01N 33/573, 33/98

US CL : 435/7.1, 7.21, 7.4

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.21, 7.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CAPLUS

search terms: ethanol, expos###, adapt###, indicat### protein#, screen###, protein kinase#, PKC#, golgi

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOCHLY-ROSEN. Localization of Protein Kinases by Anchoring Proteins: A Theme in Signal Transduction. Science. 14 April 1995, Vol. 268, pages 247-251.	1-37
X	NAGY et al. Cultured Lymphocytes From Alcoholic Subjects Have Altered cAMP Signal Transduction. Proceedings of the National Academy of Sciences USA. September 1988, Vol. 85, pages 6973-6976.	36-37
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Y		1-7, 9-15, 17, 33-35
X	MESSING et al. Chronic Ethanol Exposure Increases Levels of Protein Kinase delta and epsilon and Protein Kinase C-Mediated Phosphorylation in Cultured Neural Cells. The Journal of Biological Chemistry. 05 December 1991, Vol. 266, No. 34, pages 23428-23432.	30-32, 36-37
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Y		1-29

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 SEPTEMBER 1997

Date of mailing of the international search report

19 NOV 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ROBERT C. HAYES, PH.D.

Telephone No. (703) 308-0196



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/12980

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	HUNDLE et al. Overexpression of epsilon-Protein Kinase C Enhances Nerve Growth Factor-Induced Phosphorylation of Mitogen-Activated Protein Kinases and Neurite Outgrowth. The Journal of Biological Chemistry. 15 December 1995, Vol. 275, No. 50, pages 30134-30140.	36-37 ----- 1-31
X --- Y	MESSING et al. Protein Kinase C Participates in Up-Regulation of Dihydropyridine-Sensitive Calcium Channels by Ethanol. Journal of Neurochemistry. 1990, Vol. 55, pages 1383-1389.	36-37 ----- 1-31
X --- Y	ROIIVAINEN et al. 'Protein Kinase C and Adaption to Ethanol.' In: Toward a Molecular Basis of Alcohol Use and Abuse. 1994, pages 29-38.	36-37 ----- 1-31